See also: **II/Affinity Separation. II/Affinity Separation:** Affinity Membranes; Affinity Partitioning in Aqueous Two-Phase Systems; Aqueous Two-Phase Systems; Biochemical Engineering Aspects; Covalent Chromatography; Dye Ligands; Hydrophobic Interaction Chromatography; Immobilized Boronates and Lectins; Immobilized Metal Ion Chromatography; Immunoaffinity Chromatography; Imprint Polymers; Rational Design, Synthesis and Evaluation: Affinity Ligands. **Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Proteins. Essential Guides for Isolation/ Purification of Immunoglobulins. Appendix 2/Essential Guides to Method Development in Affinity Chromatograhy.**

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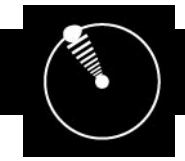
CENTRIFUGATION



Analytical Ultracentrifugation

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Analytical ultracentrifugation (AUC) involves the measurement of the radial concentration gradients of molecules created by the application of centrifugal force. In contrast to preparative centrifugation, which is used to fractionate mixtures, AUC is a purely analytical technique. Since the pioneering work of Svedberg and associates in the 1920s, AUC has been employed to characterize the mass, size, shape and association properties of macromolecules in solution. The technique has been broadly applied to research problems in biochemistry, molecular biology and polymer sciences and has also found practical applications in the pharmaceutical and biotechnology industries. Some of the most attractive features of AUC are:

1. Versatility: a wide variety of samples can be examined by AUC, including molecules ranging in size from sucrose to virus particles.
2. Rigor: AUC experiments are directly interpreted in the context of thermodynamic and hydrodynamic theory, so it is not necessary to run standards to calibrate each experiment. Also, because the experiments are performed in free solution there are no complications due to interactions with matrices or surfaces that can complicate interpretation of other types of measurements.
3. Convenience: recently, new instrumentation (Beckman Coulter XL-A and XL-I) and data analysis methods have made AUC much more convenient and accessible to the general biochemistry and polymer science communities. In contrast to earlier instruments, experiments are easy to set up and centrifugation parameters and data acquisition are all under computer control. In addition, powerful desktop computers and new software have greatly accelerated the data analysis process and have also extended the capabilities of AUC.

A complete treatment of the theory and applications of AUC is beyond the scope of this article, and the interested reader is referred to the Further Reading section.

Theoretical Background

The analytical ultracentrifuge is used to perform two different types of experiments, referred to as sedimentation velocity and sedimentation equilibrium. Sedimentation velocity is a hydrodynamic technique and is sensitive to both the mass and shape of a macromolecule. It can be used qualitatively to

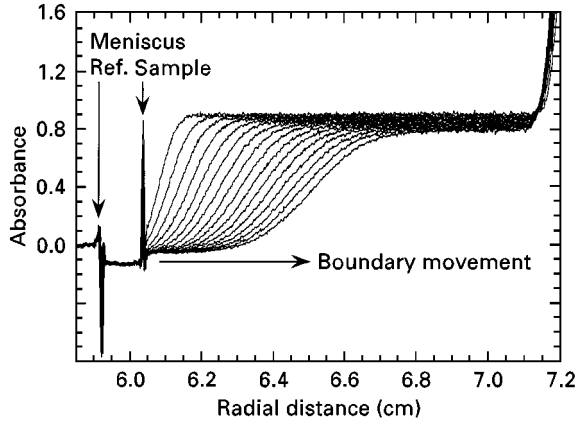

Figure 1 Sedimentation velocity of HIV-1 integrase catalytic core domain. Protein concentration 5.4 mg mL^{-1} in 20 mmol L^{-1} Tris, pH 7.5, 500 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA, 5 mmol L^{-1} DTT. Data obtained at 4°C at a rotor speed of 50 000 rpm. Radial absorption scans are recorded at 250 nm at 5 min intervals. The rate of movement of the boundary is determined by the sedimentation coefficient, *s*, and the spreading of the boundary reflects the diffusion constant, *D*.

characterize sample homogeneity and quantitatively to define mass and shape parameters of the molecular species present in a sample. The experiments are based on simple physical principles. Application of a strong centrifugal field (high rotational velocity) leads to the net movement of solute molecules away from the air–solvent interface (the meniscus) and towards the bottom of the cell, giving rise to a moving boundary (**Figure 1**). Radial scans are recorded at regular time intervals, and the data are analysed to determine both the rate of movement and broadening of the boundary as a function of time. For a homogeneous sample, a single boundary forms; for mixtures, either a single or multiple boundaries may be resolved. In quantitative terms, the rate of sedimentation of a macromolecule, $v = \mathrm{d}r/\mathrm{d}t$, is proportional to the force $\omega^2 r$, where r is the radial distance from the centre of rotation, t is time, ω is the rotational velocity. The ratio $v/\omega^2 r$ is defined as the sedimentation coefficient, s. The sedimentation coefficient has the units of time, and is expressed in Svedberg (S) units ($1\ \mathrm{S} = 10^{-13}$ s). The sedimentation coefficient may depend on concentration so it is customary to extrapolate s to zero concentration, to give s^0. In addition, to allow comparison of sedimentation coefficients obtained in different solvents and at different temperatures, s^0 is usually corrected to standard conditions (pure water at 20°C) using the following equation:

$$s^0_{20,\mathrm{w}} = s^0\left(\frac{(1-\bar{v}\rho)_{20,\mathrm{w}}}{(1-\bar{v}\rho)_{\mathrm{T,b}}}\right)\left(\frac{\eta_{\mathrm{T,b}}}{\eta_{20,\mathrm{w}}}\right) \qquad [1]$$

where $\bar{v}$ is the partial specific volume of the solute, ρ is the density of the solvent and η is the viscosity of the solvent. The subscript 20,w refers to properties measured at 20°C in water, and subscript T,b refers to properties measured at temperature T in a buffer solution b.

The sedimentation coefficient is related to molecular properties according to the following equation:

$$s = \frac{M(1-\bar{v}\rho)}{N_0 f} \qquad [2]$$

where M is the molecular mass, f is the frictional coefficient (which is related to macromolecular shape and size), and N_0 is Avogadro's number. The solvent parameters ρ and η are experimentally measurable or can be calculated from the solvent composition using tabulated data. For proteins, $\bar{v}$ can be calculated with reasonable accuracy from the amino acid composition. Any further interpretation of the sedimentation coefficient requires an independent way to measure either M or f. Fortunately, the frictional coefficient is available from the sedimentation velocity data itself. During a veloity run the boundary not only moves towards the cell bottom but also becomes broader due to diffusion. Thus, in addition to measurement of the sedimentation coefficient, s, sedimentation velocity data can also be analysed to obtain the diffusion constant, D. According to the Einstein relationship, the diffusion constant is inversely proportional to the frictional coefficient:

$$D = \frac{kT}{f} \qquad [3]$$

where k is the Boltzmann constant and T is the absolute temperature. Combining eqns [3] and [4] one obtains the Svedberg equation:

$$\frac{s}{D} = \frac{M(1-\bar{v}\rho)}{RT} \qquad [4]$$

where R is the gas constant. Thus, measurement of both s and D for a homogeneous sample in a sedimentation velocity experiment provides an independent method of obtaining the molecular mass. Given the mass, the frictional coefficient contains information about the shape and hydration of the molecule. Traditionally, frictional properties have been interpreted by modelling a macromolecule as a hydrated ellipsoid. However, more detailed, structure-based hydrodynamic calculations of frictional properties can now be readily performed using bead models.

In contrast, to sedimentation velocity, sedimentation equilibrium is a thermodynamic technique that is

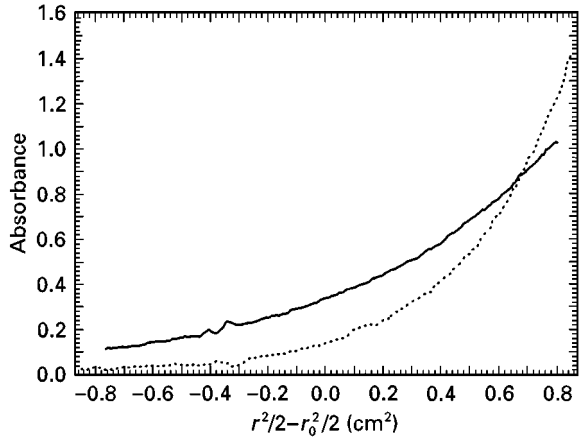

Figure 2 Sedimentation equilibrium of a 13 base pair DNA sequence. Continuous line, single-stranded; dotted line, double-stranded. 10 mmol L^{-1} Tris, pH 7.5, 50 mmol L^{-1} NaCl, 15 mmol L^{-1} KCl, 0.1 mmol L^{-1} EDTA, 2 mmol L^{-1} Spermidine. Data obtained at 4°C at a rotor speed of 40 000 rpm. Radial absorption scans recorded at 260 nm. The molecular mass of the duplex DNA is twice that of the single-stranded form.

sensitive to the mass but not the size or shape of a macromolecule. Equilibrium sedimentation is a rigorous and very accurate method of determining the molecule mass and association state of macromolecules. It is also one of the best methods of defining reversible interactions of macromolecules in solution. Sedimentation equilibrium is performed at lower rotor speeds than sedimentation velocity experiments. When the centrifugal force is sufficiently small, the process of diffusion significantly opposes the process of sedimentation and a stable, smooth, equilibrium concentration distribution of macromolecules will eventually be obtained throughout the cell (**Figure 2**). For an ideal, homogeneous macromolecule, the radial equilibrium distribution is a simple exponential function of the buoyant mass of the macromolecule, $M(1-\bar{\nu}\rho)$:

$$c(r) = c_0 \exp[M(1-\bar{\nu}\rho)\omega^2(r^2-r_0^2)/2RT] \quad [5]$$

where $c(r)$ is the sample concentration at radial position r and c_0 is the sample concentration at an arbitrary reference radial distance r_0. Deviations from the simple exponential behaviour described by eqn [5] can result from the presence in the sample of either multiple noninteracting or interacting macromelecular species, or thermodynamic nonideality. For heterogeneous, polymeric systems, various molecular weight averages (M_n, M_w and M_z) are obtained by appropriate transformations of the data and are used to assess polydispersity and self-association behaviour. In the context of protein biochemistry, the data are usually analysed in terms of discrete oligomeric species, and equilibrium AUC is an excellent method to determine the native association state of proteins. In the case where discrete oligomeric species are in reversible equilibrium, the stoichiometries (N), equilibrium constants (K_{eq}) and even the thermodynamic parameters (ΔH, ΔS) that define the interactions can be obtained using appropriate data analysis methods.

Instrumentation and Experimental Considerations

In addition to the drive system common to all ultracentrifuges, the analytical ultracentrifuge contains optical detection systems capable of directly measuring the sample concentration inside the centrifuge cell as a function of radial distance during sedimentation (**Figure 3**). The data can be viewed, or even analysed, in real time as the experiment progresses. The Beckman Coulter XL-A uses an absorbance optical system based on a xenon flashlamp and a scanning monochromator that allows measurement of sample concentration at wavelengths ranging from 200 to 800 nm (**Figure 3A**). More recently, Rayleigh interference optics were added, creating an analytical ultracentrifuge, the XL-I, that can simultaneously record data using both optical systems (**Figure 3B**). The Rayleigh interference optical system measures sample concentration based on refractive index changes. Each optical system has certain advantages and disadvantages. Absorption optics are particularly sensitive for detection of macromolecules containing a strong chromophore. Also, for samples containing two or more components with different absorption spectra (i.e. protein and nucleic acids), data can be obtained at multiple wavelengths during the same experiment to selectively monitor each chromophore. The Rayleigh interference optical system is used to analyse macromolecules lacking convenient chromophores (e.g. polysaccharides), as well as samples that contain strongly absorbing buffer components. It is also the optical system of choice for characterizing very concentrated samples. The data from each cell are acquired simultaneously on a CCD camera by the interference optical system, and the resulting rapid collection of large amounts of data is especially useful for certain types of sedimentation velocity experiments (see below). Interference optics are also useful for sedimentation equilibrium experiments that require a higher radial resolution than is provided by the absorbance optical system.

In AUC, the samples are contained in specialized cells which consist of a centrepiece containing channels to hold sample and reference solutions sandwiched between two quartz or sapphire optical windows. The optical pathlength is determined by the thickness of the centrepiece, and is typically 1.2 or

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0.3 cm. Sample requirements are fairly modest and preparation is straightforward. It is a nondestructive technique, so the sample can be recovered following the experiment. For sedimentation velocity experiments a two-channel centrepiece is typically used (**Figure 4**A) and sample volumes of $\sim 420\,\mu L$ are required. For sedimentation equilibrium experiments, the time to achieve equilibrium is inversely proportional to the square of the height of the sample column, and it is advantageous to use shorter columns. A commonly used centrepiece for this experiment contains three pairs of sample and reference channels requiring about 110 μL to produce a 3 mm column (**Figure 4**B). The sample concentrations used depend on the nature of the macromolecule that is being examined, the sensitivity of the optical system and the analysis method. For charged macromolecules, the ionic strength should be at least $50\,mmol\,L^{-1}$ to avoid nonideality due to charge effects. Samples should be equilibrated with buffer using either dialysis or gel filtration, and the equilibration buffer should be loaded into the reference sector. Analytical centrifuge rotors are available that hold either three or seven cells together with a reference cell used for radial calibration purposes (four-hole and eight-hole rotors, respectively).

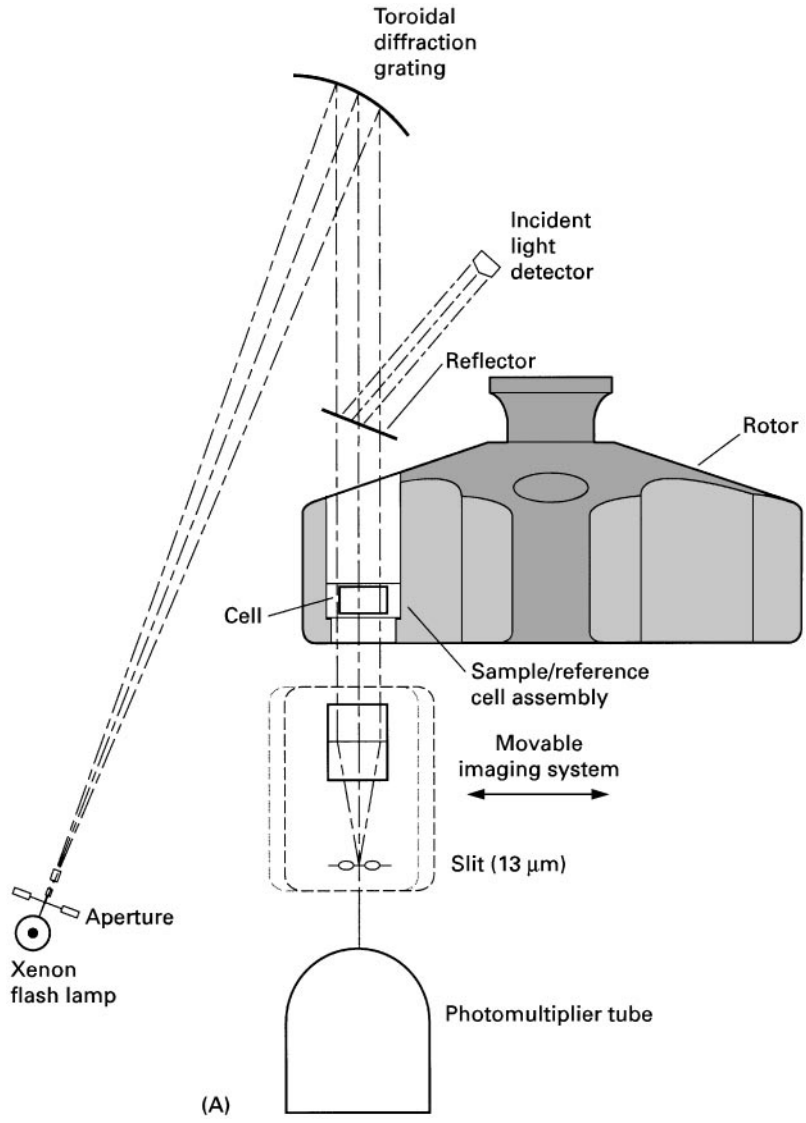

Figure 3 Optical systems in the Beckman XL-I analytical ultracentrifuge. (A) Absorption system. Light from a xenon flash lamp passes through a grating monochromator and is directed on to a sample/reference cell assembly. The transmitted light passes through a movable slit assembly and is detected with a photomultiplier tube. (B) Interference system. 675 nm light from a laser passes through a pair of slits and on to the sample and reference sectors. A series of lenses and mirrors combine the image of the sample and reference sectors to produce a fringe pattern which is imaged on a CCD camera. Refractive index changes result in vertical displacement of the fringe pattern.

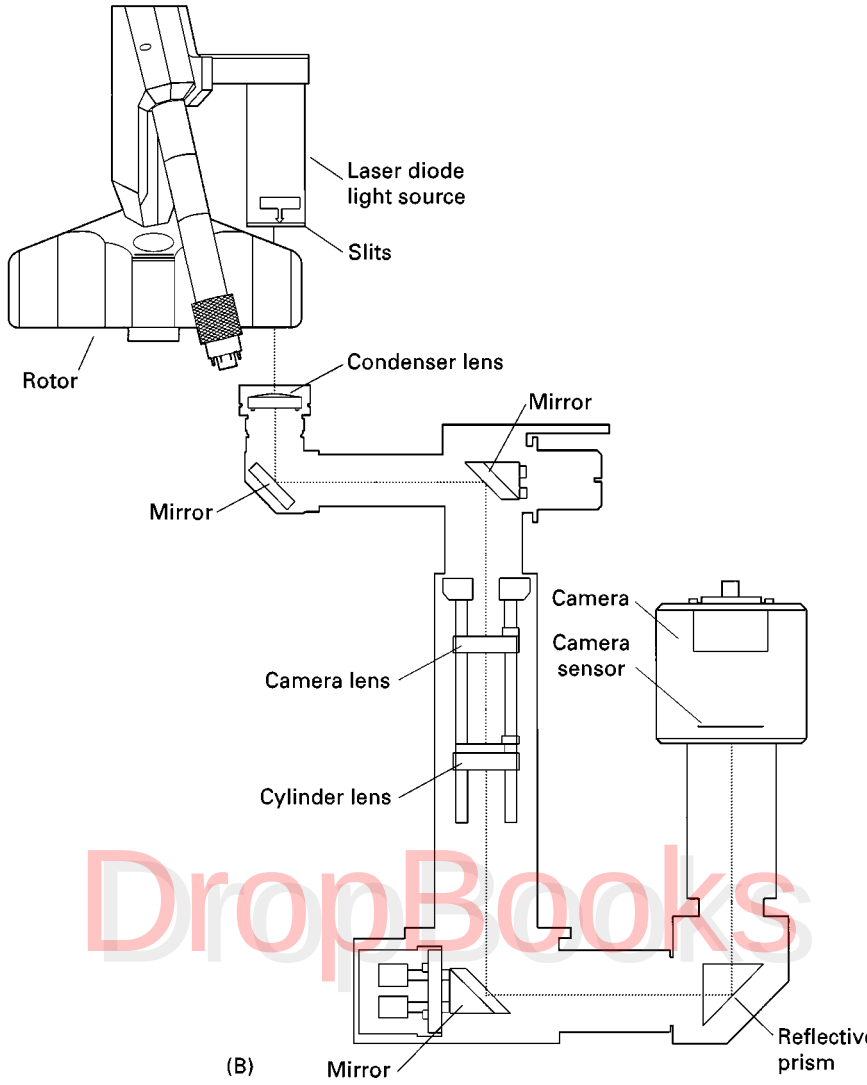

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Figure 3 *Continued*

Data Analysis Methods

In order to extract the rich information that is available from AUC experiments it is necessary to use the appropriate data analysis methods. In recent years, new approaches have been developed for the analysis of both sedimentation velocity and equilibrium data. Many of these methods are implemented in software that can be downloaded over the Internet or in commercially available packages. Although much of the analysis software is deceptively simple to use, the fitting algorithms are often complicated. In order to obtain physically meaningful parameters from analysis of AUC data it is important for the user to have a good understanding of the underlying principles along with an appreciation of the limitations in the fitting procedures.

Sedimentation Velocity

In the case of a simple, homogeneous macromolecule, analysis of sedimentation velocity data provides s, the sedimentation coefficient, and D, the diffusion constant. Under favourable conditions, it is possible to extract s and D for mixtures of non-interacting macromolecular species, provided that boundaries for each species can be resolved or deconvoluted. The situation is more complicated for reversibly associating mixtures, since it is generally not possible to assign individual boundaries to discrete species.

A traditional method for analysis of sedimentation velocity experiments is to plot the natural logarithm of the boundary position versus time. The slope of this line is proportional to $\omega^2 s^*$ where s^* represents

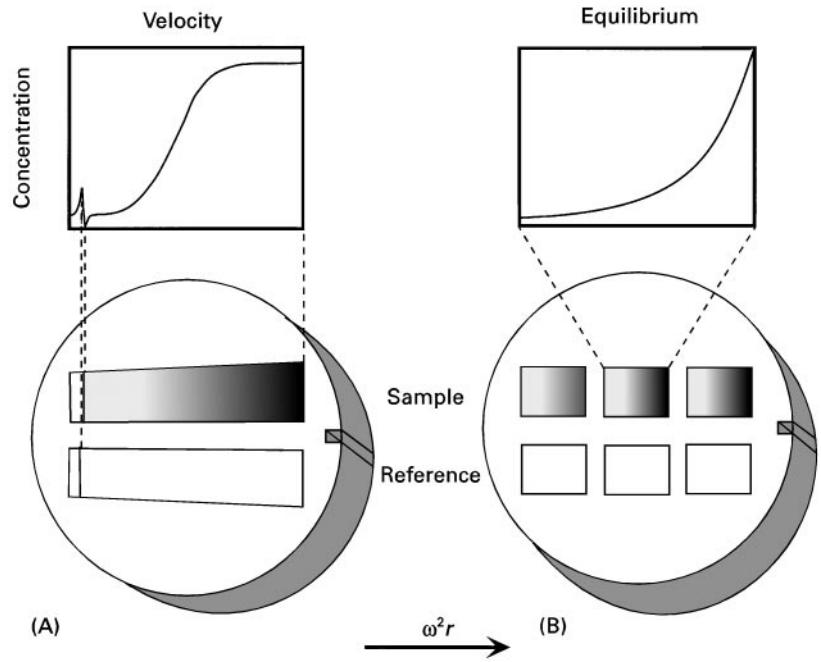

Figure 4 Sedimentation velocity and equilibrium cell designs. (A) Two-sector velocity cell. Sample is loaded into the upper sector and reference solution (buffer) is loaded into the bottom sector. The sample is centrifuged at high rotational velocity, generating a boundary that moves towards the bottom of the cell. (B) Six-channel equilibrium cell. Three sample reference pairs are loaded into the cell, which is centrifuged at moderate rotational velocity, resulting in equilibrium concentration gradients in each sample channel.

an apparent average sedimentation coefficient. This approach does not provide information about the homogeneity of the sample or the diffusion constant. Consequently, a number of analysis methods have been developed that involve analysis of the entire boundary region. In 1978, van Holde and Weischet described a transformation procedure for removing the effect of diffusion from the boundary. It is particularly useful to determine homogeneity and to detect nonideal behaviour. More recently, Stafford described a time-derivative method for analysis of velocity data in which the time-invariant noise is removed by a subtraction procedure, resulting in a great increase in the signal-to-noise ratio. This approach is particularly useful in the analysis of data obtained with the interference optics, making it feasible to work at very low protein concentrations (e.g. 10–100 $\mu g\ mL^{-1}$). Finally, there are methods for analysis of velocity experiments that involve directly fitting the scan data using either approximate or numerical solutions to the Lamm equation to determine both s and D. The Lamm equation is the partial differential equation that describes transport of solute(s) in the sector-shaped cells used in sedimentation velocity experiments. This approach can be used to fit data using single or multiple species models. Recently, Schuck has described a fitting algorithm which removes the radially-invariant and time-invariant noise contribution from the data, which makes this method particularly useful for data obtained with the interference optics.

Sedimentation Equilibrium

As in the case of sedimentation velocity, methods for analysis of sedimentation equilibrium data can be divided into model-independent and model-dependent approaches. Model-independent methods are most useful to survey sample properties qualitatively, or for analysis of complex samples, i.e. polymeric mixtures, that cannot easily be described in a model-dependent analysis. In contrast, model-dependent analysis involves direct fitting of the sedimentation equilibrium concentration gradients to relevant physical models (e.g. single ideal species, noninteracting mixtures or a reversible association). This method provides the best-fit values and the associated statistical uncertainties in the fitting parameters (e.g. molecular mass, oligomer stoichiometry and association constants) and a statistical basis to discriminate among alternative physical models.

The simplest model-independent approach to obtain the molecular mass, M, is to plot ln c versus r^2. According to eqn [5], the slope of this line is equal to $M(1 - \bar{\nu}\rho)\omega^2/2RT$. Although linearity of this plot has been taken as evidence that a sample contains a single ideal species, this method can be quite insensitive to heterogeneity, particularly if the concentration

gradient is shallow. Additionally, $d(\ln c)/dr^2$ can be calculated on a point-by-point basis to create a plot of the apparent weight-average molecular weight ($M_{w,app}$) versus concentration. For a homogeneous sample, $M_{w,app}$ will be constant as a function of concentration. An increase in $M_{w,app}$ with concentration indicates mass action-driven association. In this case, it is useful to overlay on the same plot data obtained from several samples over a range of loading concentrations and/or rotor speeds. For a reversibly self-associating system, all of the data will lie on a smooth curve, whereas for a noninteracting or slowly equilibrating system, the data will give rise to a family of nonsuperimposable curves. Other molecular weight averages (M_n, M_z) can also be obtained and can be useful in the analysis of associating systems or polymeric mixtures.

In model-dependent methods, a single experiment concentration gradient, or preferably, multiple concentration gradients, are fit to a physically relevant model using a nonlinear least-squares algorithm. In the simplest case of a single ideal species, data are fit to eqn [5]. For samples where there are more than one species in solution, or if thermodynamic nonideality is appreciable, it is necessary to fit the data to functions containing additional terms to incorporate sample heterogeneity, equilibrium association reactions or virial coefficients. Often it is difficult to distinguish between several models that fit the data equally well. In these cases, it is often useful to employ global methods in which multiple data sets that are collected over a wide range of sample loading concentrations and rotor speeds are simultaneously fit to a specific model. This global fitting approach helps to ensure that a unique solution is obtained and greatly reduces the statistical uncertainty in the parameters. Global nonlinear least-squares fitting of sedimentation equilibrium data was originally implemented in the NONLIN algorithm, and now several programs are available. In addition, equilibrium data are often fit using models programmed by the user within a general-purpose data analysis package.

Conclusions

AUC is a robust and widely accepted analytical method to characterize the molecular mass, size, shape and association of molecules in solution. It has been used extensively by biochemists and molecular biologists to define properties of biological macromolecules and has also found applications within the polymer science community. Experiments are performed using specialized centrifuge cells in an analytical ultracentrifuge capable of measuring radial concentration gradients using absorption or refractometric optics. In sedimentation velocity experiments a moving boundary forms upon application of a high centrifugal force. The rate of movement of the boundary is determined by the sedimentation coefficient and the broadening of the boundary with time occurs because of diffusion. The sedimentation coefficient is a function of a molecule's mass and frictional properties whereas the diffusion constant in only determined by the frictional properties. Sedimentation equilibrium measurements are performed at lower rotation velocities where the sedimentation force is balanced by diffusion. The shape of the concentration gradient is determined by the molecular weight of the species present in the sample. A variety of computer data analysis methods have been developed for both sedimentation velocity and equilibrium data.

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